Detection of *Campylobacter fetus* in Artificial Insemination Bulls with a Transport Enrichment Medium

M.M. Garcia, G.M. Ruckerbauer, M.D. Eaglesome and W.E. Boisclair*

ABSTRACT

One hundred and five bulls from an artificial insemination unit were tested for the presence of *Campylobacter fetus* subspecies *venerealis*. The method involved the inoculation of preputial samples into a new transport enrichment medium prior to culture and immunofluorescence tests. Seventeen bulls (16%) were found to be either positive or suspected carriers of *C. fetus* at one or more sampling times. The average age of these 17 bulls was about two years greater than the average age of all the bulls in the unit. A combined treatment of vaccination and dihydrostreptomycin sulfate injection suppressed or eliminated the organism from carrier bulls. The use of transport enrichment medium has increased our capability and effectiveness to monitor the presence of *C. fetus* in artificial insemination bulls.

Key words: *Campylobacter fetus*, campylobacter infections, transport enrichment medium for *Campylobacter fetus* isolation.

RÉSUMÉ

Cette étude consistait à rechercher la présence de *Campylobacter fetus*, sous-espèce *venerealis*, chez 105 taureaux d'un centre d'insémination artificielle. Les auteurs inoculèrent à cette fin des échantillons prépu-tiaux dans un nouveau milieu de transport enrichi, avant de les soumettre à la culture et à l'immunofluorescence. Ils constatèrent que 17, i.e. 16% de ces taureaux étaient des porteurs réels ou suspects de *C. fetus*, au moment d'un ou de plusieurs tests. Leur âge moyen dépassait de deux ans celui de tous les autres taureaux du centre. Une intervention qui impliquait la vaccination et l'injection de sulfate de dihydrostreptomycine supprima ou élimina le microbe, chez les porteurs. L'utilisation d'un milieu de transport enrichi a par conséquent amélioré la possibilité de surveiller plus efficacement la présence de *C. fetus*, chez les taureaux des centres d'insémination artificielle.

Mots clefs: *Campylobacter fetus*, infections à *Campylobacter*, milieu de transport enrichi pour l'isolement de *Campylobacter fetus*.

INTRODUCTION

The problems associated with the survival of *Campylobacter fetus* subsp. *venerealis* during transport of samples from the field to the laboratory have always been a major consideration in the diagnosis of campylobacteriosis (vibriosis) by cultural methods. Recently, Clark and Dufty (1) introduced a transport enrichment medium which enhanced the recovery of *C. fetus* from preputial samples. The medium, made up of solidified bovine serum and several microbial inhibitors, was contained in vials which were provided with a microaerophilic atmosphere. This transport enrichment medium (TEM) was evaluated by Winter and Caveney (2) who found it suitable for the successful isolation of *C. fetus* subsp. *venerealis* with an inoculum as small as 100 organisms. Transport enrichment medium has also been recommended for use in the examination of semen (3). Garcia *et al* (4) used TEM to advantage in diagnosing *C. fetus* infection of both cows and bulls in areas where refrigeration of samples during transport posed a major problem. In a more recent quantitative evaluation of TEM (unpublished) we found that *C. fetus* subsp. *venerealis* can increase a thousandfold while being incubated at 37°C in TEM for four days.

This report describes our experience in the monitoring of bulls for campylobacteriosis in an artificial insemination (AI) unit over a two-year period. To our knowledge, TEM had not been evaluated in Canadian AI testing programs prior to this study.

MATERIALS AND METHODS

AI UNIT

The AI unit under study is a well established centre which maintains the bulls in individual stalls in a clean environment. The AI unit also employs several means of

*Agriculure Canada, Animal Diseases Research Institute, NEPEAN, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9 (Garcia, Ruckerbauer and Eaglesome) and Agriculture Canada, District Office, Animal Health Division, Box 578, Brockville, Ontario K6V 5V7 (Boisclair).

Submitted November 10, 1982.

minimizing contamination during semen and preputial sample collections. At the time of entry of a bull into the semen collection area, the preputial orifice and the ventral skin surface are disinfected. The hind quarters of all teaser animals are also disinfected. During semen collection, new disposable gloves are used by handlers during collection from each animal.

In the two years prior to this study, none of the bulls in this AI stud were found to be carriers of *C. fetus*. During this period, neither vaccination nor antibiotic injection was employed in conjunction with annual tests for campylobacteriosis. Specimens for *C. fetus* culture were not inoculated into transport media but were transported directly to the laboratory, under refrigerated condition, within the same day. Laboratory processing of samples for *C. fetus* was done within 24 hours after sampling. The cultural procedure employed followed that of Ruckerbauer *et al* (5).

MONITORING OF AI BULLS

Preparation of TEM — Transport enrichment medium was prepared according to the method described by Clark and Dufty (1). The basic medium consisted of 1 mL bovine serum, 100 units polymixin B, 50 μg brilliant green, 300 μg 5-fluorouracil, 3 μg nalidixic acid and 100 μg cycloheximide. The first two ingredients were obtained from Grand Island Biological Co., New York, N.Y. and the rest from SIGMA, St. Louis, Missouri. The medium was gelled in a boiling water bath for 2 min; cooled and then flushed with a special gas mixture (3.5% O_2, 10% CO_2 and 86.5% N_2) to provide the microaerophilic atmosphere needed for *Campylobacter* growth. Vials of TEM were maintained in the refrigerator for at least one week prior to use.

Collection of preputial samples — The present study involved the monitoring of one hundred and five bulls ranging in age from 11 months to 16 years. Initial sampling was timed to coincide with the annual testing of the AI unit bulls. Subsequent collections within the year were made from culture positive or FAT positive or suspicious bulls after four, six, eight, nine and ten months. Changes to the collection procedure were kept to a minimum to avoid disruption of the work-flow. The only change from the previous practice was the introduction of TEM for transporting the preputial samples. Briefly, the sampling method was as follows: Preputial smegma were collected using plastic pipets and cannulae (6). The smegma was washed off with 4 mL of saline into a sterile vial. After allowing epithelial cells to settle for 20 minutes, 2 mL of the upper liquid layer were carefully withdrawn using a sterile syringe and needle. One mL of the liquid was injected into each of two vials of TEM. The sample was well mixed with the TEM by shaking the vial. The inoculated vials were placed in boxes insulated with styrofoam so that they were transported to the laboratory within a temperature range of 18-37°C. Samples were received in the laboratory on the same day of collection.

Laboratory tests — Immediately on arrival, inoculated TEM vials were incubated at 37°C for four to five days. Then a sterile swab was dipped into the inoculated TEM vial and applied to a small area of the surface of each of duplicate cystine heart blood agar plates containing 2 U/mL polymyxin B, 2 μg/mL novobiocin and 20 μg/mL cycloheximide. Streaking of the inoculum over the entire surface of the plates was completed with a flamed wire loop. All plates were incubated at 37°C for four to six days under a microaerophilic atmosphere. The identification of *C. fetus* was based on cultural and biochemical tests (3,7) and by the direct fluorescent antibody test (FAT) as described by Ruckerbauer *et al* (5). In the latter, a minimum of three microscope slides were prepared from each bacterial plate. Organisms were removed from the plate by sweeping a sterile loop across the area of growth. The loopful of material was suspended in 1 mL distilled water, thoroughly mixed and one drop placed onto a slide without spreading. The preparation was air dried and stained immediately or stored at 4°C until required. Fluorescent antibody test results were reported as positive, negative or suspicious (5).

Treatment of carrier bulls — Carrier bulls were vaccinated by subcutaneous injections of 2 mL of Vibrin,[1] a formolized *C. fetus* bacterin in an oil adjuvant. Most of the carrier bulls received three doses of the vaccine (see Table I for details). The initial dose was given on the third month (or three months after *C. fetus* infection was discovered) with booster injections on the tenth and 23rd months. Three days prior to each sampling on the eighth to the 12th month carrier bulls were injected with dihydrostreptomycin sulfate[2] by the intramuscular route at the rate of 22 mg per kg body weight.

RESULTS

MONITORING OF AI BULLS

Of 105 bulls tested, 17 (16%) were found to be either positive or suspected carriers of *C. fetus* at one or more sampling time (Table I). The 17 bulls included 12 culture positive and five culture negative but FAT positive. At the first sampling date, ten bulls were found to be culture positive and four bulls were considered suspicious by FAT. Four months after the discovery of infection and one month after the first dose of vaccine was given, seven of the original ten culture positive bulls remained infected and two previously culture negative bulls became positive. Three of the four initially

[1]Vibrin, Norden Laboratories, Lincoln, Nebraska.
[2]Ethamycin, Roger/STB, London, Ontario.

TABLE I. Laboratory Test Results for the Presence of *C. fetus* in Bulls from an AI Unit

Animal	Age (years)	Months of testing							
		0	4	6	8	9	10	12	24
A1	9	+	+	FA	—	—	—	—	—
A2	6	+	—	—	—	—	—	—	R
A3	10	S	+	+	—	—	—	—	R
A4	8	+	+	+	—	—	—	—	—
A5	4	S	—	—	—	—	—	—	—
A6	2	S	—	—	—	—	—	—	FA
A7	7	+	+	+	—	—	—	—	—
A8	7	+	—	—	—	—	—	—	R
A9	13	+	+	FA	—	—	—	—	—
A10	6	+	+	—	—	—	—	—	R
A11	3	S	—	—	—	—	—	—	—
A12	7	+	+	+	—	—	—	—	R
A13	12	+	+	R					
A14	6	—	+	+	—	—	FA	—	—
A15	7	+	returned to private service and brought back to AI					—	—
A16	3	—						—	FA
A17	6	—						—	FA

+ : positive on culture, positive on FAT
— : negative on culture, negative on FAT
FA: negative on culture, positive on FAT
S : negative on culture, suspicious on FAT (ten typically fluorescing organisms per slide, nonspecific staining present)
R: removed from AI unit prior to this sampling period
Vaccination schedule:
A1-A10, A16 and A17 — vaccinated on third, tenth and 23rd month
A11 and A14 — vaccinated on tenth and 23rd month
A13 and A15 — received only one dose on third and 23rd month, respectively.

FAT suspicious animals turned FAT negative. A marked decrease (50%) in culture positive bulls was observed at the sixth month sampling. However, only one (A10) of these had become cleared of the infection after the fourth month sampling. Moreover, bulls A1 and A9 were still FAT positive. By the eighth month, soon after dihydrostreptomycin sulfate injection, all previously positive bulls at the AI unit became negative by culture and FAT. Except for A14 (tenth month) and A6 (24th month) which showed positive FAT reaction, all previous carrier animals remained negative. It should be noted that no antibiotic was administered after the 12th month. Of interest are bulls A16 and A17 which did not appear to be *C. fetus* carriers until the 24th month.

Local swellings occurred around the injection site of vaccinated bulls particularly after the booster injections. These were considered unsightly and resembled closely Mitchell's (8) observation of persistent local reactions which consisted of granulomatous lesions with necrosis and giant cell formation.

The ages of the positive carrier bulls ranged from 6-13 years with an average of about eight years. Together with the FAT suspicious bulls, the average age was about seven years. In both cases, the average age of the carrier bulls was much higher than that of all the bulls in the AI Unit (five years).

TRANSPORT ENRICHMENT MEDIUM

With the introduction of TEM, we observed at least a twofold increase in the capability to process samples compared to earlier methods. During annual testing of the AI unit, two samplings a week at an interval of three days (e.g. Monday and Thursday) were most suitable for laboratory processing of the preputial samples.

Transport enrichment medium appeared to increase the reliability and ease of recovering *C. fetus* as the numbers of contaminating organisms growing on the inoculated cystine-heart agar plates were reduced. Furthermore, polymixin B in TEM may have inhibited the isolation of *C. sputorum* subsp. *bubulus*, a saprophytic *Campylobacter* common in the reproductive tract of cattle, thus reducing the time to process the preputial samples. However, an occasional problem associated with contaminant overgrowth was encountered. This could have resulted from improper sampling of the preputial material, or the presence of environmental materials (e.g. sawdust, straw, etc.) in the prepuce which contained organisms resistant to the level of inhibitory substances present in TEM. In such a case, repeat samples were requested.

DISCUSSION

In this study, we attempted to address two major areas concerning *C. fetus* in AI bulls: a) the need for improved detection and monitoring systems and b) the problem of eliminating the organism in carrier bulls. The data presented indicate that the presence of *C. fetus* carrier bulls in AI units may be a chronic problem which requires a vigorous monitoring and control program. With the adoption of improved techniques such as the use of TEM, a more accurate picture of the infection status of an animal with respect to *C. fetus* can be obtained. Moreover, the application of selective transport media such as TEM almost automatically increases the capability of the laboratory to handle specimens for *C. fetus* testing since the pressing need to culture the samples upon arrival has been obviated. As more studies to find better media for *C. fetus* recovery are continuing, improvements in sampling techniques have not been overlooked. We have retained the Bartlett's pipette and collection technique (6) since it has been reported to be superior to preputial washing for collecting samples for bacterial examination (9). Of interest is a recent report (10) indicating that further improvement in the sampling technique to recover *C. fetus* can be attained. This involves using a "scraper" to obtain

smegma from the preputial and penile mucosa. However, owners of highly prized bulls may resist this method. It is evident that further improvements in the sampling and culture techniques together with a better systematic monitoring schedule will lead to a more effective AI testing program.

The second area of concern is more difficult to answer as we are dealing with an organism (*C. fetus* subsp. *venerealis*) which is not only recognized for its specialized adaptation to the bovine reproductive environment (11) but also for its capability to change antigenically within the host (12). The fact that *C. fetus* subsp. *venerealis* persisted in several bulls despite the strict hygienic measures and vaccination employed by the AI unit leads one to speculate on whether the organism has become more strongly adherent to the preputial tissues or, in some ways, protected from direct action by antibodies and thus have become increasingly difficult to eliminate. The detection of FAT positive organisms in treated bulls by the tenth (A14) and 24th months (A6, A16 and A17), respectively, despite vaccination and antibiotic treatments, appear to support this concern.

The process of adaptation or persistent colonization is more likely to occur in older bulls. Wagner *et al* suggested (13) that as the bull ages, the penile mucosa becomes irregular with more and deeper crypts which may facilitate the establishment and persistence of *C. fetus*. An analysis of the age of the positive and suspected carrier bulls indicates an average of about seven years. This is higher than the average age of all the bulls tested (five years). Moreover, three bulls (A5,A6 and A11) less than five years were found negative at the second sampling after having been initially FAT suspicious. These observations lend support to the opinion that bulls six years or over are more likely to be *C. fetus* carriers than younger ones (14,15,16, 17). Another factor related to age is the probability that the longer a bull is kept in the stud the more likely it is to be exposed to infection by *C. fetus*. However, Bier *et al* (18) observed that experimentally infected bulls of two age groups (41-49 months and 66-74 months) did not develop agglutinating antibodies to *C. fetus* in preputial cavities.

Following the first dose of *C. fetus* vaccine, two positive (A2,A7) and three suspected (A5,A6,A11) carrier bulls became negative for *C. fetus*. This clearance may be due to vaccination although spontaneous recovery from infection cannot be discounted. There is considerable evidence that vaccination possesses both preventive and therapeutic value (19,20,21,22). Vaccination of female cattle has been known to produce a degree of protection similar to the natural immunity resulting from campylobacteriosis. In vaccinated bulls, a challenge inoculum instilled into the preputial cavity disappears rapidly within 24 hours by a yet unknown mechanism (10). Clark *et al* (20) observed that positive bulls vaccinated twice, five weeks apart, with a killed *C. fetus* subsp. *fetus* bacterin, in a mineral oil adjuvant, eliminated infection within two weeks after the second injection. Bouters *et al* (19) found that 30 out of 41 infected bulls (70%) were free of *C. fetus* organisms within 42 days. The mean interval between vaccination and disappearance of the organism was 30.6 days. The remaining 11 bulls resistant to the first vaccination responded much earlier (average of 12 days, range 5-21 days) after a booster injection was given. In the present study, the second and third doses of vaccine were injected long after the bulls became culture negative and were given with the intention of protecting against *C. fetus* reinfection.

In practice, not all commercially prepared vaccines produce the same level of protection as do experimental vaccines. Several factors such as the type of vaccine, route and schedule of administration, virulence and quantity given are important considerations. Berg and Firehammer (23) stressed that vaccinal antibody titres can decrease rapidly after vaccination. However, Clark and Dufty (24) showed that bulls can be protected against *C. fetus* infection for up to two years with subcutaneous injections of a mineral-oil adjuvant vaccine containing 20 mg dry wt each of *C. fetus* subsp. *venerealis* and biotype *intermedius*. They suggested that biannual booster vaccination should be adequate for maintaining the immunity. Cameron (25) obtained higher antibody titres in heifers with a trivalent vaccine compared to a monovalent commercial product. An effective vaccination program should therefore assure a high degree of and prolonged immunity to protect bulls from infection.

Three days after antibiotic treatment, all previously positive bulls became negative on culture and FAT. Considering the relative sensitivity of the culture method employed, we suspect that some bulls had either become cleared of infection prior to antibiotic treatment or that the treatment eliminated the organism or reduced its numbers to a level not detectable by culture or FAT. Seger *et al* (26) observed that campylobactericidal levels were maintained in the animal for four days after a single subcutaneous dose of 22 mg dihydrostreptomycin sulfate per kg of body weight. They further claimed that the subcutaneous (systemic) route results in a longer exposure of the sheath to streptomycin, eliminated in the urine, than preputial infusion or intramuscular injection of the antibiotic. Lein *et al* (16) observed that carrier bulls treated with dihydrostreptomycin sulfate were negative two, four and six weeks after treatment. However, no sampling was done earlier than two weeks after treatment. Despite the effectiveness of antibiotic treatment in clearing up *C. fetus* infection, reports of the appearance of streptomycin-resistant mutants of *C. fetus* (27,28) and the increased susceptibility to reinfection of previously treated bulls (26) should serve to caution against relying mainly on this measure as a permanent solution for the eradication of *C. fetus* infection in AI units. It must be emphasized that, in this study, the interval between

antibiotic injection and sampling was too short to allow proper evaluation of this treatment in eliminating *C. fetus*. A more appropriate step is to allow a longer period between treatment and sampling or to obtain multiple samples at different time intervals between two weeks to six months after the last treatment (18).

The possible inhibitory effect of residual antibiotic in the preputial samples on the cultural recovery of *C. fetus* was considered during this work. Unpublished data from trials with A.D.R.I. and AI bulls indicated that the effect of streptomycin injection was mainly on the *C. fetus* population in the animals and that no residual antibiotic was detected in the samples that might have affected cultural recovery of the organism.

ACKNOWLEDGMENTS

We are grateful to Dr. K.L. Malkin, W. Alton Boyd, Maria Horvath, Janice Moe, Ruth Robertson and Blake Stewart for their assistance in various aspects of the study.

REFERENCES

1. **CLARK BL, DUFTY JH.** Isolation of *Campylobacter fetus* from bulls. Aust Vet J 1978; 54: 262-263.
2. **WINTER AJ, CAVENEY NT.** Evaluation of a transport medium for *Campylobacter (Vibrio) fetus*. J Am Vet Med Assoc 1978; 173: 472-473.
3. **STALHEIM OHV, BARTLETT DE, CARBREY EH, KNUTSON WW, LANGFORD EV, SEIGFRIED L,** eds. Recommended procedures for the microbiologic examination of semen. Proc Am Assoc Vet Lab Diagnost 1978; 21: 515 (Suppl) 1-20.
4. **GARCIA MM, EAGLESOME MD, HAWKINS CF, ALEXANDER FCM.** Campylobacteriosis in Jamaican cattle. Vet Rec 1980; 106: 287-288.
5. **RUCKERBAUER GERDA M, MALKIN K, MITCHELL D, BOULANGER P.** Vibriosis: Demonstration of *Vibrio fetus* and *Vibrio bubulus* organisms in preputial fluid by immunofluorescence and cultural techniques. Can J Comp Med 1974; 38: 321-327.
6. **BARTLETT DE.** Procedures for diagnosing bovine venereal trichomoniasis and handling infected herds. J Am Vet Med Assoc 1949; 114: 293-305.
7. **SMIBERT RM.** Genus II Campylobacter Sebald and Veron 1963, 907. In: Buchanan RE, Gibbons NE, eds. Bergeys manual of determinative bacteriology. Baltimore: Williams and Wilkins, 1975: 207-212.
8. **MITCHELL D.** Some effects of experimental *Vibrio fetus* (venerealis) infection on cattle inoculated with a commercial bacterin. Can J Comp Med 1968; 32: 474-479.
9. **DUFTY JH, McENTEE K.** Evaluation of some culture media and sampling techniques for the diagnosis of vibriosis in the bull. Aust Vet J 1969; 45: 140-144.
10. **TEDESCO LF, ERRICO F, DEL BAGLIVI LP.** Comparison of three sampling methods for the diagnosis of genital vibriosis in the bull. Aust Vet J 1977; 53: 470-472.
11. **VERON M, CHATELAIN R.** Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. Int J Sys Bact 1973; 23: 122-134.
12. **CORBEIL LB, SCHURIG GGD, BIER PJ, WINTER AJ.** Bovine venereal vibriosis. Antigenic variation of the bacterium during infection. Infect Immun 1975; 11: 240-244.
13. **WAGNER WC, DUNN HO, VAN VLECK LD.** Incidence of vibriosis in an AI stud. Cornell Vet 1965; 55: 209-220.
14. **ADLER HC, LINDEGAARDE LE.** Bovine genital vibriosis: eradication from Danish A.I. centers. Nord Vet Med 1965; 17: 237-241.
15. **HUGHES DE.** Notes on vibriosis, with special reference to the isolation of *Vibrio fetus* from semen and preputial fluids. Cornell Vet 1956; 46: 249-256.
16. **LEIN D, ERICKSON I, WINTER AJ, McENTEE K.** Diagnosis, treatment and control of vibriosis in an artificial insemination center. J Am Vet Med Assoc 1968; 153: 1574-1580.
17. **PLASTRIDGE WN, WALKER EC, WILLIAMS LF, STULA EF, KIGGINS EM.** Isolation of *Vibrio fetus* from bulls. Am J Vet Res 1957; 18: 575-578.
18. **BIER PJ, HALL CE, DUNCAN JR, WINTER AJ.** Experimental infections with *Campylobacter fetus* in bulls of different ages. Vet Microbiol 1977; 2: 13-27.
19. **BOUTERS R, DEKEYSER J, VANDEPLASSCHE M, VANAERT A, BRONE E, BONTE P.** *Vibrio fetus* infection in bulls: curative and preventative vaccination. Br Vet J 1973; 129: 52-57.
20. **CLARK BL, DUFTY JH, MONSBOUGH MJ.** Vaccination of bulls against bovine vibriosis. Aust Vet J 1968; 44: 530.
21. **CLARK BL, DUFTY JH, MONSBOROUGH MJ, PARSONSON IM.** Immunisation against bovine vibriosis. Vaccination of bulls against infection with *Campylobacter fetus* subsp. *venerealis*. Aust Vet J 1974; 50: 407-409.
22. **CLARK BL, DUFTY JH, MONSBOROUGH MJ, PARSONSON IM.** Studies on venereal transmission of *Campylobacter fetus* by immunised bulls. Aust Vet J 1975; 51: 531-532.
23. **BERG RL, FIREHAMMER BD.** Effect of interval between booster vaccination and time of breeding on protection against campylobacteriosis (vibriosis) in cattle. J Am Vet Med Assoc 1978; 173: 467-474.
24. **CLARK BL, DUFTY JH.** The duration of protection against infection with *Campylobacter fetus* subsp. *venerealis* in immunized bulls. Aust Vet J 1982; 58: 220.
25. **CAMERON CM.** Antibody response of guinea pigs and cattle to a *Campylobacter fetus* oil emulsion vaccine. Onderspoort J Vet Res 1982; 49: 177-179.
26. **SEGER CL, LANK RB, LEVY HE.** Dihydrostreptomycin for treatment of genital vibriosis in the bull. J Am Vet Med Assoc 1966; 149: 1634-1639.
27. **MORGAN WJB.** The sensitivity of *Vibrio fetus* to streptomycin and the emergence of resistant mutants. J Gen Microbiol 1958; 19: 517-521.
28. **VANDEPLASSCHE MA, FLORENT A, BOUTERS R, HUYSMAN A, BROONE E, KEKEYSER P.** The pathogenesis, epidemiology and treatment of *Vibrio fetus* infection in cattle. Compt Rend Recherches 1963; 1-85.